DOI: 10.1002/chem.200902833

Poly(arginine)-Selective Coprecipitation Properties of Self-Assembling Apoferritin and Its Tb^{3+} Complex: A New Luminescent Biotool for Sensing of Poly(arginine) and Its Protein Conjugates

Hiroshi Tsukube,* Yuki Noda, and Satoshi Shinoda[a]

Abstract: The apoferritin protein and apoferritin–Tb^{3+} complex were demonstrated to form oligomeric and polymeric self-assemblies in neutral aqueous solutions, based on characterization by using luminescence and UV/Vis spectroscopy, dynamic light scattering, and transmission electron microscopy. Addition of a 20-mer or higher poly(arginine) to the solution resulted in coprecipitation through nanoscale interactions, while biological proteins and other poly(amino acids) rarely yielded precipitates under the conditions employed. The apoferritin–Tb^{3+} complex assembly exhibited a particularly long-lived green luminescence in aqueous solution, and its poly(arginine)-selective precipitation behavior was followed by monitoring the changes in luminescence. The poly(arginine)-tagged albumin precipitated selectively and quantitatively, so that the apoferritin–Tb^{3+} complex can function as a new luminescent biotool for the sensing of poly(arginine) and its protein conjugates.

Keywords: ferritin · lanthanides · luminescence · poly(arginine) · terbium

Introduction

Apoferritin is the protein component of the ferritin iron-storage protein, which consists of 24 subunits that form a spherical hollow shell with external and internal diameters of 13 and 7.5 nm, respectively. The apoferritin shell has eight 0.4 nm diameter hydrophilic channels for the passage of metal ions and small molecules.[1] The inner cavity of apoferritin has recently been used as a nanoscale molecular vessel in the generation of non-iron clusters, noncovalent incorporation of organic guest molecules, and compartmentalization of drugs and magnetic resonance imaging reagents.[2] The parent ferritin protein has been well characterized and forms a two-dimensional ordered self-assembly on silicon substrates and in single-walled carbon nanotubes.[3] Apoferritin also acts as a protein ligand of the Tb^{3+} cation and exhibits long-lived green luminescence in aqueous solution.[4] Several luminescent lanthanide complexes have recently received attention for medical and bioanalytical applications.[5] Hildebrandt et al. combined several Tb^{3+} complexes with quantum dots for time-resolved fluoroimunoassays. Sames et al. developed armed cyclen–lanthanide complexes as near-infrared emissive sensors that function in aqueous solution. Vogel et al. reported lanthanide-based resonance energy transfer for high-throughput screening of biomolecules. Although the number of synthetic lanthanide complexes that exhibited intense emissions in aqueous media still remains limited,[6] apoferritin and other protein derivatives have potential as supramolecular ligands for luminescent lanthanide complexation.[4,7]

We demonstrate that self-assembling apoferritin and the apoferritin–Tb^{3+} complex offer selective coprecipitation with poly(arginine) and their protein conjugates. Protein–protein interactions are central to many biological and bioapplication processes, and the precipitation method has been widely used in the characterization of nanoscale interactions and the fractionalization of targeted proteins.[8] We found that ferritin derivatives exhibited unique self-assembling properties in aqueous solutions and formed coprecipitates with specific polycations (Figure 1). In particular, the apoferritin–Tb^{3+} complex has several outstanding features as an effective luminescent biotool for such protein sensing: 1) the complex maintains the nanoscale hollow shell featuring anionic amino acid residues and is available for further self-assembly; 2) excitation of protein chromophores provides long-lived green luminescence even in aqueous

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Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/chem.200902833.

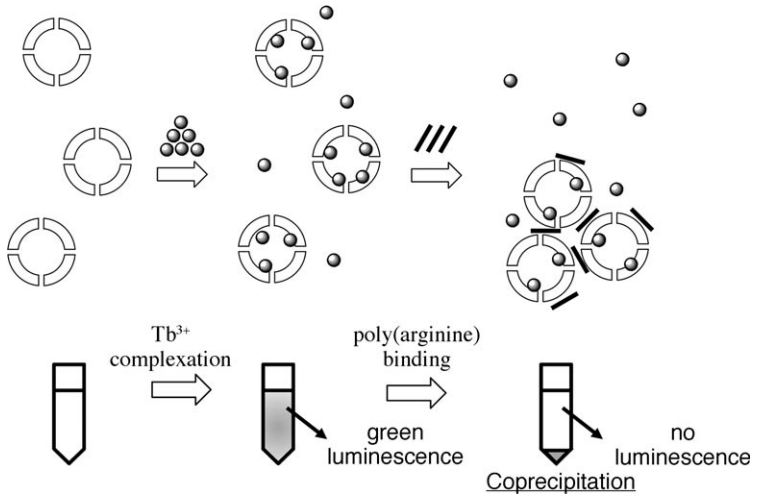

Figure 1. Self-assembly, Tb^{3+} complexation, and protein coprecipitation profiles of apoferritin.

media;[4] 3) several functional groups and metal cations can be introduced to modify the protein surface; and 4) some derivatives were reported to be internalized into the cells.[9] Furthermore, with a diameter of 13 nm the apoferritin–Tb^{3+} complex has a much larger polyanionic surface than common spherical proteins with diameters of 2~5 nm. Since the negatively charged domains are arranged in a mosaic-like fashion, its surface characteristics are expected to exhibit unique selectivity in polycation recognition and coprecipitation processes. Indeed, apoferritin and the apoferritin–Tb^{3+} complex offered the selective formation of coprecipitates with a series of poly(arginine) substrates, the amounts of which were significantly dependent on the chain lengths of the substrates. Although biological proteins and other synthetic poly(amino acids) rarely yielded precipitates with apoferritin and its Tb^{3+} complex, the poly(arginine)-tagged protein formed a precipitate. Such ploy(arginine)-selective precipitation phenomena were detected not only spectroscopically, but also visually when the luminescent apoferritin–Tb^{3+} complex was employed. Thus, the present results successfully reveal that the apoferritin–Tb^{3+} complex can function as a new biotool for luminescence sensing of poly(arginine) and its protein conjugates.

Results and Discussion

Self-assembling characteristics of apoferritin and apoferritin–Tb^{3+} complex: Commercially available ferritin (Sigma–Aldrich, Japan) solution was dialyzed against 0.1 M 3-(*N*-morpholino)propanesulfonic acid (MOPS) buffer (0.3 wt% $Na_2S_2O_5$, 0.15 M NaCl, pH 7.0) containing 2,2′-bipyridine (5×10^{-5} M) to remove Fe^{3+} cations, and then against NaCl (0.15 M) solution, as previously described.[10] The resulting apoferritin solution exhibited identical ultraviolet (UV) absorption and circular dichroism (CD) spectra to those previously reported.[4] Inductively coupled plasma-atomic emission spectrometry (ICP-AES) revealed that less than 0.6 Fe^{3+} cation was included in one protein subunit. Addition of $TbCl_3$ salt to the resulting apoferritin solution readily yielded the apoferritin–Tb^{3+} complex, which exhibited characteristic luminescence signals at around 490, 545, 590 and 620 nm (Figure S1 in the Supporting Information). A decay analysis of the observed luminescence signals indicated two components with lifetimes of 0.4 and 0.8 ms, respectively. The complexation and luminescence profiles were similar to those previously reported,[4] indicating that two or three Tb^{3+} cations occupy the Fe^{3+} binding sites of each protein subunit under the conditions employed. In the following experiments, a MOPS buffer solution (0.1 M, pH 7.0) containing apoferritin (5×10^{-7} M), $TbCl_3$ (7.2×10^{-5} M, 6.0 equiv per protein subunit), and NaCl (0.15 M) was prepared, and mechanically stirred for 3 h. After centrifugal separation (3000 rpm or 640 *g* for 10 min) of insoluble materials, such as hydrolyzed metal species and large protein aggregates, the apoferritin–Tb^{3+} complex solution included >90% apoferritin and >3 equiv Tb^{3+} cations per protein subunit.

The self-assembling properties of apoferritin and its Tb^{3+} complex were characterized by dynamic light scattering experiments. The effect of Tb^{3+} addition on the size distribution of apoferritin is illustrated in Figure 2. Although Cd^{2+} and Fe^{3+} cations have been reported to promote the two-dimensional crystallization of apoferritin,[11] the addition of

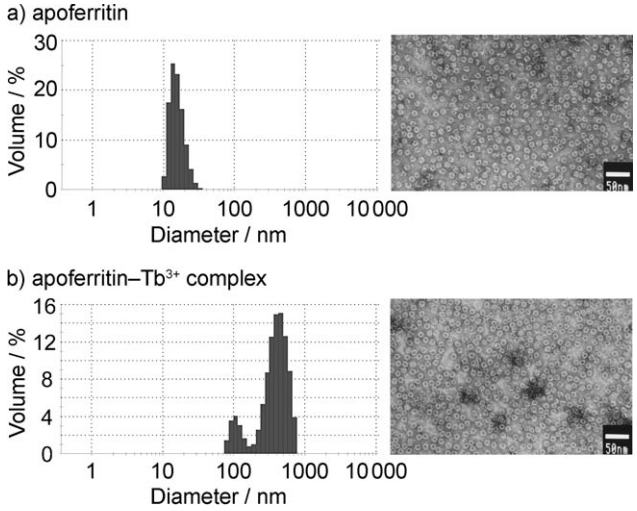

Figure 2. Effect of Tb^{3+}complexation on volume distribution and TEM image of the apoferritin self-assembly. The statistics was converted from the intensity distribution obtained by dynamic light scattering measurements; scale bar: 50 nm.

Tb^{3+} cations to the aqueous solution produced higher oligomers with diameters of about 100 and 400 nm. The zeta-potentials of these two self-aggregates were measured as an indication of the surface electrical properties of the protein aggregate.[12] Both aggregates of apoferritin and its Tb^{3+} complex exhibited similar zeta-potentials close to zero (−7.6 and −8.8 mV, respectively) while they had different size-distribution patterns.

We first established the conditions to obtain the apoferritin aggregate with high purity. As previously reported,[11] apoferritin was confirmed to exist as mainly monomeric, dimeric, and trimeric aggregates in neutral aqueous solutions. This is an equilibrium process in which the aggregate states are highly sensitive to the concentration of the protein and the nature of buffer solution, coexisting species and other environmental factors. Our dynamic light scattering characterization revealed that the obtained aggregate occupied more than 99.5% of the total volume (Figure 2a). Thus, we employed this in the following coprecipitation experiments. Transmission electron microscopy (TEM) observations also confirmed that they formed higher aggregates on the carbon grids, which further suggests that the addition of Tb^{3+} cations enhanced the ordering of the protein self-assembly on a solid matrix. Ultrastructural analysis of the TEM images showed that both apoferritin derivatives have spherical shapes, the size of which (diameter = ca. 12–13 nm) was similar to the reported values.[1]

Coprecipitation profiles of poly(arginine): A series of poly(arginines) were first examined in coprecipitation experiments with apoferritin derivatives based on the following reasons.[13] High numbers of arginines are often involved in protein–protein interactions; the attachment of poly(arginine) to the targeted protein promoted internalization more effectively than poly(lysine); and poly(arginine)-tag technology is also employed in protein purification and detection processes.

When poly(L-arginine) was added to an aqueous solution of apoferritin or its Tb^{3+} complex at neutral pH, the apoferritin derivative precipitated and the amount of precipitate was significantly dependent on the chain length of the added poly(arginine) (Figure 3). Combined analysis by using UV/Vis, ICP and time-of-flight mass spectrometry (TOF-MS) revealed that the precipitate contained apoferritin, Tb^{3+} cations and poly(arginine). When 1 equiv of poly(L-arginine) 20-mer per protein subunit was added, then typically approximately 80% of the apoferritin was precipitated, based on the UV spectral change at 280 nm. A 0.1 M HCl solution of the dissolved precipitate had an intense TOF-MS peak at 3142 due to the added poly(arginine) 20-mer (Figure S2 in the Supporting Information). L-Arginine has a permanent positive charge on its side chain, but rarely yielded precipitates with apoferritin and its Tb^{3+} complex. The poly(arginine) 10-mer formed a coprecipitate with apoferritin modestly, and did not provide reproducible data with the apoferritin–Tb^{3+} complex, indicating that the 10-mer has not sufficient chain length to interact with the apoferritin protein. In contrast, the 20-mer and larger poly(L-arginines) formed insoluble materials in which apoferritin was quantitatively included. Since the poly(D-arginine) 20-mer exhibited similar precipitation behavior to the poly(L-arginine) 20-mer, the longer poly(arginine) chain is essentially involved in an electrostatic interaction with the apoferritin surface, and the stereochemistry of the arginine unit had no signifi-

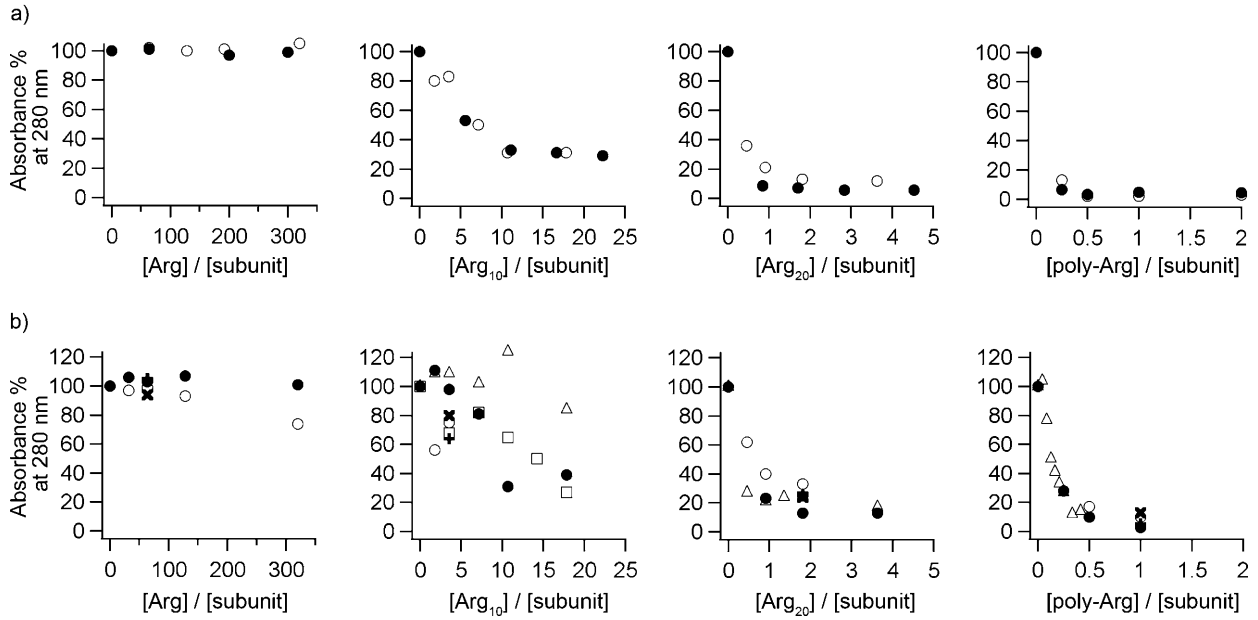

Figure 3. Effect of chain length of poly(L-arginine) on coprecipitation with: a) apoferritin, and b) the apoferritin–Tb^{3+} complex. Results of several independent experiments are shown (each run is shown by different symbols: ○, ●, ×, +, △, and □). Arg, Arg_{10}, and Arg_{20} are arginine and its 10- and 20-mer, respectively. Poly-Arg is estimated as a 64-mer from the average molecular weight.

cant influence on the precipitation process. Modeling calculations by using AMBER version 7.0 suggested that the extended "rod" conformations of the poly(arginine) 10- and 20-mer have chain lengths of 3.3 and 6.6 nm, respectively. These poly(arginines) are too large to penetrate the protein channel; therefore, they interact with the outer surface on the apoferritin shell and promote precipitation.

Coprecipitation profiles of poly(amino acids): In addition to poly(arginine), cationic poly(L-lysine), neutral poly(L-asparagine) and anionic poly(L-aspartic acid) were examined with regard to coprecipitation with the apoferritin–Tb^{3+} complex; their molecular weights ranged from 4000 to 15000. These poly(amino acids) have no absorption around 280 nm and the precipitated amounts were directly determined by the absorbance changes of the supernatants at 280 nm. Only poly(lysine) formed a precipitate with the apoferritin–Tb^{3+} complex, while the other polymers did not have effective interaction with the apoferritin derivatives, as indicated in Figure 4. The precipitation efficiency of poly(lysine) was lower than that of poly(arginine) (Figure 3), which confirms that the polycationic moieties located on the rigid "rod" peptide skeleton play an important role in the precipitation process. These phenomena were followed by monitoring the green luminescence of the apoferritin–Tb^{3+} complex (Figure 5).

Coprecipitation profiles of biological proteins and the poly(arginine) conjugate: Biological proteins were also examined as substrates in coprecipitation experiments; these included albumin from bovine serum, myoglobin from equine skeleton muscle, α-chymotrypsin from bovine pancreas, cytochrome c from bovine heart, and lysozyme from chicken egg white. One equivalent of each protein substrate per ferritin subunit was added to apoferritin or the apoferritin–Tb^{3+} complex buffered solution at pH 7.0. After being stirred for 3 h at room temperature, centrifugal separation was carried out at 3000 rpm for 10 min. Each protein substrate had an intense absorption at 280 nm; therefore, the absorption spectrum of the supernatant was compared with the calculated spectrum produced by summing of spectra of the protein substrate and apoferritin with the corresponding concentration (Figure 6a). Table 1 summarizes the coprecipitation results with the apoferritin–Tb^{3+} complex, together with their molecular weights (M_W) and isoelectric point (pI) values. Of the substrates, cytochrome c and lysozyme have positively charged surfaces at neutral pH, but produced no precipitate with the apoferritin–Tb^{3+} complex. It is well known that these proteins have "patch" structured polycations on their surfaces, in which several positive amino acid residues are closely located. These proteins have spherical shapes with diameters of approximately 4 nm, so that the arrangement of the positive charges on the polymer substrate was confirmed as the most essential factor in the promotion of coprecipitation. Although other biological proteins did not interact with the apoferritin protein, the albumin conjugate linked with the poly(L-arginine) 10-mer effectively formed a precipitate (Figure 6b). Sodium dodecyl sulfate polyacrylamide gel electropho-

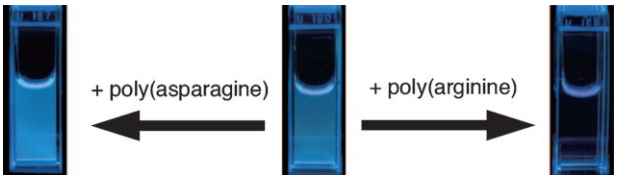

Figure 5. Pictures of the luminescent apoferritin–Tb^{3+} complex (center) and after the addition of poly(asparagine) and poly(arginine).

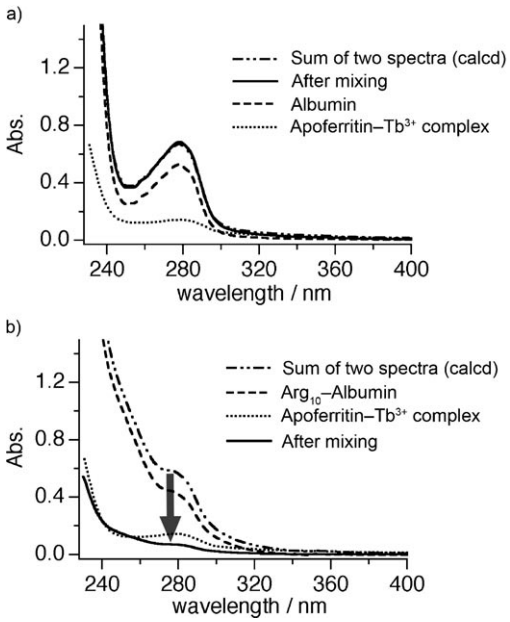

Figure 6. Changes in UV/Vis absorption of the apoferritin–Tb^{3+} complex after the addition of: a) bovine serum albumin, and b) bovine serum albumin conjugated with poly(arginine) 10-mer. The calculated spectra were produced by summing the absorption spectra of the two components.

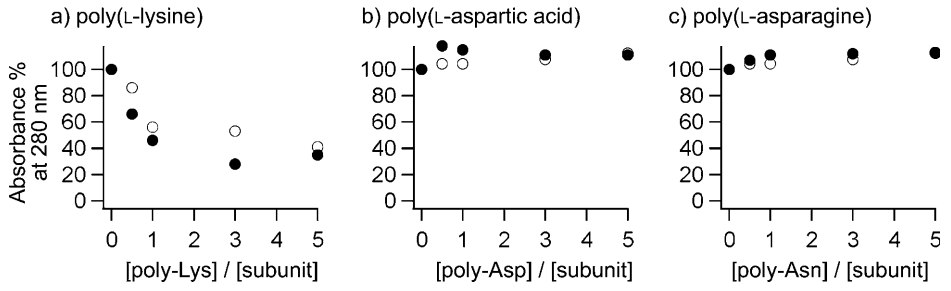

Figure 4. Coprecipitation of poly(amino acids) with the apoferritin–Tb^{3+} complex. Two different runs are shown by ● and ○.

Table 1. Coprecipitation profiles of biological proteins with apoferritin and the apoferritin–Tb^{3+} complex.

Protein	pI	M_W [kD]	Absorption [%] at 280 nm[a]	
			apoferritin	Tb^{3+} complex
albumin	5.4	66.5	103	101
myoglobin	7.5	17.0	99	98
α-chymotrypsin	8.4	25.3	99	97
cytochrome c	9.3	12.3	97	103
lysozyme	11.0	14.3	102	101

[a] Percentage was determined by the change in absorbance of apoferritin or the apoferritin–Tb^{3+} complex at 280 nm after mixing and centrifugation.

resis (SDS-PAGE) analysis of this conjugate suggested that three poly(arginine) 10-mers were incorporated in one albumin protein (Figure S3 in the Supporting Information). The absorption spectrum measured after mixing 0.5 equiv of poly(L-arginine)-conjugated albumin per subunit of the apoferritin–Tb^{3+} complex showed a decrease in absorbance at 280 nm to 25% from the total sum of the absorbances of the two proteins. The unmodified albumin and poly(arginine) 10-mer were not suitable substrates individually; therefore, two or three units of the poly(arginine) 10-mer moieties acted cooperatively in the precipitation process.

Conclusion

The selective coprecipitation of poly(arginine) and protein conjugates with self-assembling apoferritin and the apoferritin–Tb^{3+} complex was successfully demonstrated and provided useful application in protein detection and separation. As illustrated in Figure 5 (center), the apoferritin–Tb^{3+} complex exhibited a green emission upon excitation with a laboratory UV lamp (254 nm, 4 W). Coprecipitation occurred rapidly when poly(arginine) was added to the aqueous solution, and the resulting supernatant was nonluminescent (Figure 5; right). Poly(asparagine) and other polymer substrates formed no precipitate and had no luminescence response (Figure 5; left). We compared rod-like rigid poly(arginine), relatively flexible poly(lysine) and spherical proteins with highly charged patches as polycationic substrates. Among them, the employed ferritin derivatives particularly offered coprecipitation with the poly(arginine) 20-mer with 6.6 nm length. Since the ferritins have negatively charged domains on the 13 nm × 13 nm scaled surfaces in a mosaic-like fashion, the nanoscale matching between polyanion and polycation moieties is thought to offer unique poly(arginine)-selective coprecipitation phenomena. Therefore, the present apoferritin–Tb^{3+} complex could operate as a new type of luminescent biotool for the detection and separation of poly(arginine) and its protein conjugates.

Experimental Section

General: Luminescence spectra were recorded on a Perkin–Elmer LS-50B luminescence spectrometer. UV/Vis spectra were obtained with a Hitachi U-3500 spectrophotometer. Dynamic light scattering experiments were performed by using a Malvern HPPS-ET high performance particle size analyzer, and TOF-MS spectra were recorded on a Shimadzu Axima CFT Plus V.2.4.0 spectrometer. The determination of Fe^{3+} and Tb^{3+} cations by using IPC was performed by Shimadzu Analytical & Measuring Center, Inc., Kyoto, Japan.

Materials: Ferritin from equine spleen (type I) was purchased from Sigma–Aldrich (Japan) and used after dialysis to remove the Fe^{3+} cations. The remaining Fe^{3+} cations in the apoferritin used were determined by ICP spectroscopic analysis. The apoferritin–Tb^{3+} complex solution was prepared by mixing apoferritin and $TbCl_3$ in MOPS buffer (0.1 M, pH 7.0) containing NaCl (0.15 M). After being mechanically stirred for 3 h, the insoluble materials were removed by centrifugal separation at 3000 rpm or 640 *g* for 10 min.

Poly(L-arginine) 10- and 20-mer were provided as TFA salts by Cosmo Bio Ind. (Japan) and their purities were confirmed as > 90% by high performance liquid chromatography (HPLC) analysis, and the poly(D-arginine) 20-mer (TFA salt) was obtained from Sigma–Aldrich (Japan). Higher molecular weight poly(L-arginine) with a mixture of molecular weights (5000–15000) was purchased form Nacalai Tesque, Inc. Poly(L-lysine), poly(L-asparagine), and poly(L-aspartic acid) were also commercially available, and had similar molecular weights to poly(L-arginine). The albumin-poly(arginine) conjugate was provided by Kokusan Chemical (Japan). SDS-PAGE analysis revealed that three of the poly(arginine) 10-mers were linked with one albumin molecule through Cys–Cys linkages (Figure S3 in the Supporting Information). All proteins were purchased from Sigma–Aldrich (Japan) and were used without further purification. Ultrapure water was used in each experiment after ion exchange (Advantec RFU-354-BA).

TEM observation: Apoferritin and the apoferritin–Tb^{3+} complex assemblies were fixed by dropping onto 400 mesh carbon grids and treatment with uranyl acetate. The grids were then dried, placed in a grid chamber, and stored in a desiccator prior to TEM observation. All TEM observations were performed by using a Jeol JEM1200EX microscope at Hanaichi Ultra Structure Research Institute (Japan).

Precipitation experiments: The coprecipitation experiments were carried out by adding a polymer substrate to a solution of apoferritin or the apoferritin–Tb^{3+} complex (5×10^{-7} M) in MOPS buffer (0.1 M) containing NaCl (0.15 M) at pH 7.0. The mixture was mechanically stirred for 3 h at room temperature, and then centrifuged at 3000 rpm for 10 min. The supernatant obtained was analyzed by using UV and luminescence spectroscopy, dynamic light scattering and ICP spectroscopy. The precipitate was dissolved in HCl (0.1 M) and then analyzed by using ICP and TOF-MS techniques.

When nonchromophoric poly(amino acids) were employed as substrates, the precipitated amounts of apoferritin derivatives were directly estimated from the change in absorbance at 280 nm of the supernatant. Although all the examined proteins have absorption bands at around 280 nm, the observed absorbance of each mixture was close to the sum of the absorbance values of apoferritin and each protein (Figure 6). Almost the same results were obtained in most cases when measured again after 1 day.

Acknowledgements

The authors thank the Kobe Particle Center of Sysmex, Inc., Japan, for measuring the zeta-potentials of apoferritin aggregates (Malvern Instruments Zetasizer Nano ZS). This work was supported by the Ministry of Education, Culture, Sports, Science and Technology, Japan (Grants-in-Aid of Science Research, Nos. 20900138 and 20245014).

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Received: October 14, 2009
Published online: March 1, 2010

